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Quantitative imaging of proteoglycan in cartilage using a gadolinium probe and microCT

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Summary

Objective: Micro-computed tomography (microCT) imaging has the potential to allow the three-dimensional (3D) visualization of cartilage morphology. However, cartilage intensity on a microCT image is weak because cartilage does not strongly attenuate X-rays. This work was designed to demonstrate that exposure of cartilage to charged gadolinium compounds modifies the intensity to allow an improved visualization of cartilage morphology and the determination of proteoglycan content.

Design: Trypsin was used to deplete proteoglycan in bovine nasal cartilage disks. Disks were then exposed to Gd^{3+}, gadopentetate ($Gd–DTPA^{2-}$), or gadoteridol (Gd–HP–DO3A), and imaged with microCT. The intensities of the disks were measured from the images and compared to the actual proteoglycan content determined with a dimethylmethylene blue assay.

Results: Treatment of naïve disks with 200 mM Gd^{3+} for 24 h at room temperature produced a 2.8-fold increase in intensity on microCT images. Similar treatment with 200 mM $Gd–DTPA^{2-}$ produced a 1.4-fold increase. After 2 h of trypsin treatment at room temperature, the intensities of cartilage disks exposed to 200 mM Gd^{3+} decreased by 12%. Conversely, the intensities of trypsin-treated disks exposed to 200 mM $Gd–DPTA^{2-}$ increased by 15%. Trypsin treatment caused a 4% increase in the intensities of disks exposed to neutral Gd–HP–DO3A. The correlation between proteoglycan content and the microCT intensity of cartilage treated with Gd^{3+} was very good ($r^2 = 0.81$).

Conclusions: Gadolinium and microCT allow an improved 3D visualization of cartilage and quantification of its proteoglycan content.

Key words: Cartilage, Gadolinium, MicroCT, Proteoglycan.

Introduction

Micro-computed tomography (microCT) is a relatively new imaging modality that has the spatial resolution to visualize and quantify morphology in small animal joints. The first generation of commercially available instruments was designed for studies of *ex vivo* samples such as bone biopsies[1]. However, second generation instruments are capable of obtaining images from living rodents with high spatial resolution in reasonable image acquisition times[2]. Most microCT studies thus far have focused on bone[3]. Reports of quantitative microCT imaging of cartilage have not appeared, possibly because the inherent intensity of cartilage in a microCT image is weak. Therefore, a means to improve the visualization of cartilage is very desirable.

One possibility for improving cartilage visualization with microCT can be discerned from the magnetic resonance imaging (MRI) literature. In 1992, Kusaka *et al.*[4] used MRI to show that the uptake of manganese (as Mn^{2+}) or gadolinium (as gadopentetate ($Gd–DTPA^{2-}$)) in cartilage is associated with the proteoglycan distribution. Since these two ions are paramagnetic, they alter the appearance of cartilage on MRI. Subsequently, other scientists investigated this phenomenon more thoroughly. Many efforts were focused on $Gd–DTPA^{2-}$ because it is not toxic at doses effective for MRI and is easily obtained as the active ingredient in the commercially available contrast agent called Magnevist®. In a seminal article published in 1996, Bashir *et al.*[5] showed that $Gd–DTPA^{2-}$ is taken up in cartilage in inverse concentration to the proteoglycan content. Laurent *et al.*[6] extended this work by showing that $Gd–DTPA^{2-}$ and MRI could measure proteoglycan depletion induced by papain in the rabbit knee.

While the paramagnetism of gadolinium makes it useful for MRI, the metal is also known to attenuate X-rays. In fact, commercially available MRI contrast agents containing gadolinium, such as Magnevist® and Gadovist®, were investigated as contrast agents for X-ray CT[7,8]. However, these studies did not address the application of contrast agents for CT of cartilage. We hypothesized that gadolinium exposure would enhance the appearance of cartilage on microCT images and allow improved visualization. Furthermore, the uptake of gadolinium would reflect the proteoglycan distribution and could be quantified from microCT images.

Method

All images were acquired with a Scanco microCT-40 X-ray tomograph (Scanco Medical, Inc., Bassersdorf,

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Switzerland) using 55 kVp energy, 145 μA current, and a 200 ms integration time. For each slice, 250 projections were acquired and reconstructed in a 1024 × 1024 matrix; the field-of-view was 16.4 mm and the voxel size was 20 μm isotropic. Bovine nasal cartilage disks, 6 mm diameter by 1 mm thick, were stored in Dulbecco's modified Eagle's medium at −20°C until the preparation for microCT. Proteoglycan depletion was produced using porcine trypsin solution (25 mg/ml; Sigma–Aldrich, St. Louis, MO). This was frozen at −20°C until needed, then warmed to 4°C overnight, then to room temperature (22°C) over the following day. Gd^{3+}, $Gd–DTPA^{2-}$ or gadoteridol (Gd–HP–DO3A) solution was prepared by diluting $GdCl_3$ (Sigma–Aldrich), Magnevist® (Berlex, Wayne, NJ) or ProHance® (Bracco, Milan, Italy), respectively, in 0.9% w/v saline (Baxter, Deerfield, IL). All solutions were maintained at room temperature.

DEMONSTRATION OF THE CONCEPT AND OPTIMIZATION OF THE GADOLINIUM CONCENTRATION

The primary goal of this part was to demonstrate the effect of proteoglycan depletion on the distribution of Gd^{3+} or $Gd–DTPA^{2-}$ in cartilage. A secondary goal was to determine a practical working concentration of gadolinium for future studies.

Thirteen groups of cartilage disks were prepared for microCT. Disks were thawed overnight at 4°C. Next, disks were pretreated by soaking in saline or trypsin solution for 24 h at room temperature with occasional swirling. Twelve groups of three or more disks were produced by removal from the pretreatment solutions and immediately transferring to solutions of one of three concentrations of Gd^{3+} or $Gd–DTPA^{2-}$ (50 mM, 100 mM, and 200 mM). One additional group was transferred to fresh saline. All disks were soaked for an additional 24 h at room temperature.

In preparation for microCT, each cartilage disk was removed from solution, blotted, and placed in a sample holder. A single slice through the middle of the disk was acquired. The mean intensity of each cartilage disk was measured using the Scanco Ximage software. An increased intensity meant that more X-ray attenuation was occurring and therefore more gadolinium was present. Intensities from three disks were obtained and averaged for each group.

COMPARISON OF PROTEOGLYCAN CONTENT AND MICROCT INTENSITY

This work was conducted to determine the relationship between the proteoglycan contents of cartilage disks and their intensities in microCT images. A second goal was to demonstrate that the charge of the gadolinium complex affects its uptake in cartilage.

For this work, the trypsin solution was diluted to 0.25 mg/ml in 0.9% w/v saline (Baxter) to allow more control over proteoglycan loss. The cartilage disks were thawed at 22°C for 80 min, and then refrigerated at 4°C for nearly 3 days. The disks were then placed in a hood for 1 h to equilibrate at room temperature (~22°C). Disks were pretreated by immersing them in the diluted trypsin solution for 0, 2, 4, or 6 h at room temperature and ensuring good mixing with an orbital shaker at 75 rpm. Next, groups of three pretreated disks were immersed in 200 mM Gd^{3+}, $Gd–DTPA^{2-}$ or Gd–HP–DO3A solutions for 24 h, again at room temperature with orbital shaking. Thus, there were 12 treatment groups of three disks each.

In preparation for microCT, each cartilage disk was removed from the gadolinium solution, blotted, and placed in a custom-built cell that allowed three disks to be scanned simultaneously. A three-dimensional (3D) scan of the disks was acquired. For each scan, a maximum of 137 slices were acquired, covering up to a 2.83-mm thick region. The maximum total image acquisition time was 34.5 min. After scanning, each disk was placed in saline in a small vial and frozen at −20°C until assessment of proteoglycan concentration.

The mean intensity of each disk was quantified using a custom software application written for the Aphelion platform (ADCIS, Caen, France). The algorithm consisted of the following steps:

(1) Read in the 16-bit 3D dataset.
(2) Make a two-dimensional (2D) MAX image that is the maximum of all slices.
(3) Threshold the MAX image to find the outline of each individual cartilage disk.
(4) Threshold each image, and then identify and obtain a count of voxels belonging to each cartilage disk.
(5) Gather detected voxel intensity information to calculate the total, average, and standard deviation of all intensities within each cartilage disk.
(6) Calculate the mean and standard deviation of the average intensities of all cartilage disks for each treatment group.

The proteoglycan content of each cartilage disk was quantified using a modification of the dimethylmethylene blue assay as described by Farndale *et al.*[9]. A 20 mM sodium phosphate buffer was adjusted to pH 6.8 with phosphoric acid. Next, 1 mM ethylenediamine tetraacetic acid (Mallinckrodt Baker, Phillipsburg, NJ) and 2 mM dithiothreitol (Sigma–Aldrich) were added, followed by the addition of a papain suspension (Sigma–Aldrich) to produce a concentration of 3 mg/ml papain. Each disk was digested in 3 ml of the papain-containing phosphate buffer by heating between 50 and 60°C for a minimum of 3 h. The digest was centrifuged to produce a supernatant. Two hundred fifty microliters of supernatant was diluted in 1 ml of a 9.295 mg/ml iodoacetic acid (Sigma–Aldrich) solution, 750 μl phosphate digestion buffer, and 3 ml of a 50 mM Tris/HCl (Sigma–Aldrich) pH 8 buffer. Standards were made by dissolving chondroitin sulfate A (Sigma–Aldrich) in phosphate digestion buffer and iodoacetic acid solution. A color reagent was made with dimethylmethylene blue (Sigma–Aldrich). Samples of the standard solutions and triplicates of the supernatant solution from each disk were pipetted onto a 96-well plate and the color reagent was added to each. Then the optical density at 525 nm was determined for each well with an UV/Vis spectrophotometer (SpectraMax Plus, Molecular Devices, Sunnyvale, CA).

Statistical comparisons of group means were performed using the one-way analysis of variance (JMP statistical analysis software, version 4.0.4, SAS Institute, Inc., Cary, NC). Significance was established at $P < 0.05$.

Results

Figure 1 consists of representative microCT images of cartilage disks that show the effects of treatment with Gd^{3+} or $Gd–DTPA^{2-}$. In the absence of gadolinium,

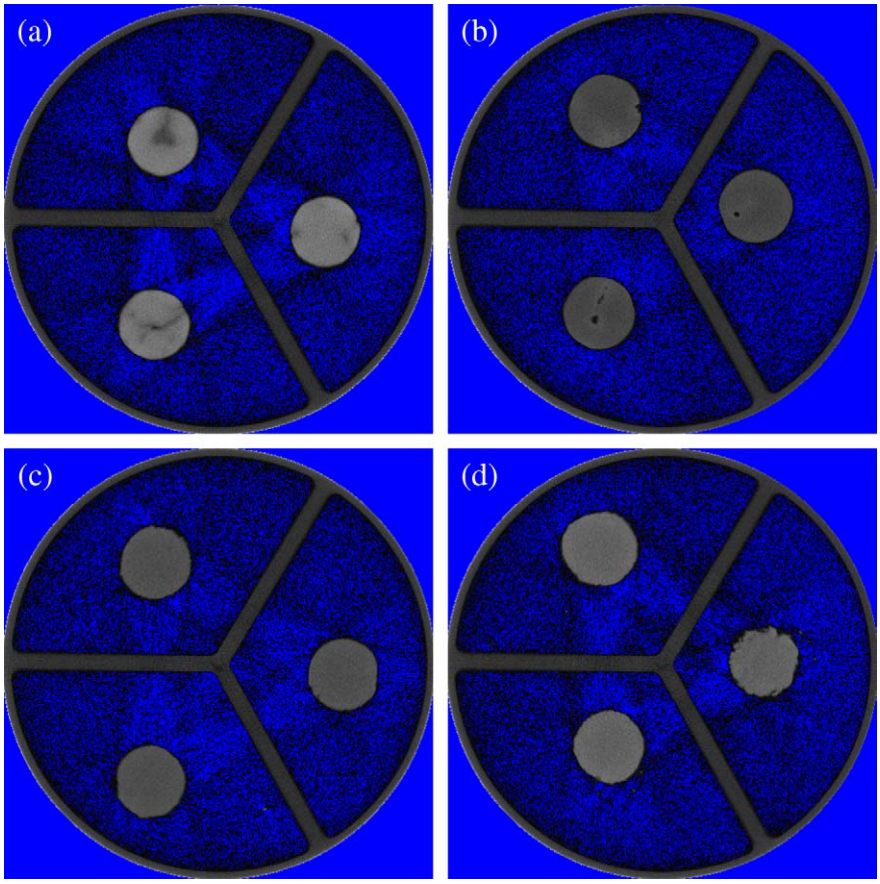

Fig. 1. MicroCT images of bovine nasal cartilage disks treated with saline or trypsin followed by Gd^{3+} or $Gd{-}DTPA^{2-}$ exposure. Trypsin removes negatively charged proteoglycans causing reduced electrostatic attraction of Gd^{3+} (darkening) or reduced electrostatic repulsion of $Gd{-}DTPA^{2-}$ (brightening). (a) Saline, day 1; 100 mM Gd^{3+}, day 2; (b) saline, day 1; 200 mM $Gd{-}DPTA^{2-}$, day 2; (c) trypsin, day 1; 100 mM Gd^{3+}, day 2; (d) trypsin, day 1; 200 mM $Gd{-}DTPA^{2-}$, day 2.

saline-treated cartilage appeared dark gray (not shown). In disks treated with saline on day 1, Gd^{3+} was taken up, causing brightening [Fig. 1(a)], whereas $Gd{-}DTPA^{2-}$ was largely repelled and the cartilage remained dark [Fig. 1(b)]. When disks were treated with trypsin, the proteoglycans were removed. Therefore, Gd^{3+} was not taken up as much and the cartilage was darker than when it was saline-treated [Fig. 1(c)]. On the other hand, removal of proteoglycans meant that more $Gd{-}DTPA^{2-}$ could enter a cartilage disk and it appeared brighter than a saline-treated disk [Fig. 1(d)].

Figure 2 shows the change in intensity with Gd^{3+} concentration for cartilage disks pretreated for 24 h with saline or trypsin solution. In cartilage pretreated with saline, increasing $[Gd^{3+}]$ increased the cartilage intensity. This behavior fit a rising exponential curve (intensity $= 2320 + 4188 \times (1 - \exp(-0.019 \times [Gd^{3+}]))$, $r^2 = 0.994$). In particular, the intensity of cartilage treated with 200 mM Gd^{3+} was 2.8-fold greater than that of naïve cartilage. In trypsin-treated disks, increasing $[Gd^{3+}]$ also increased the cartilage intensity. This fit a linear function (intensity $= 2668 + 10.95 \times [Gd^{3+}]$, $r^2 = 0.999$).

Figure 3 shows analogous results for $Gd{-}DTPA^{2-}$. Increasing $Gd{-}DTPA^{2-}$ concentration also increased cartilage intensity but to a lesser degree than Gd^{3+}. In cartilage pretreated with saline, increasing $[Gd{-}DTPA^{2-}]$ increased the cartilage intensity linearly (intensity $= 2231 + 4.78 \times [Gd{-}DTPA^{2-}]$, $r^2 = 0.963$). The intensity of cartilage treated with 200 mM $Gd{-}DTPA^{2-}$ was 1.4-fold greater than that of naïve cartilage. When

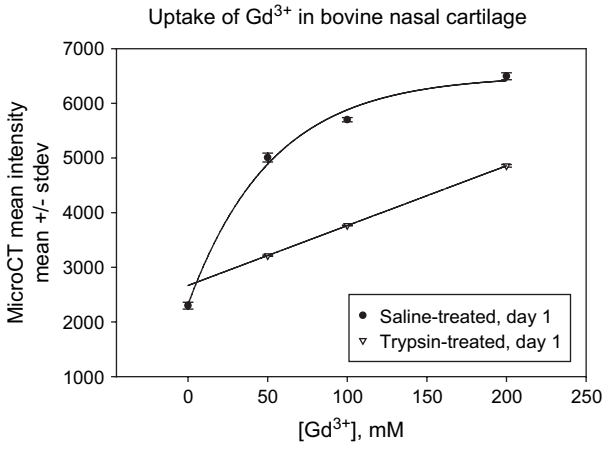

Fig. 2. Uptake of three concentrations of Gd^{3+} in bovine nasal cartilage disks after pretreatment with saline or trypsin.

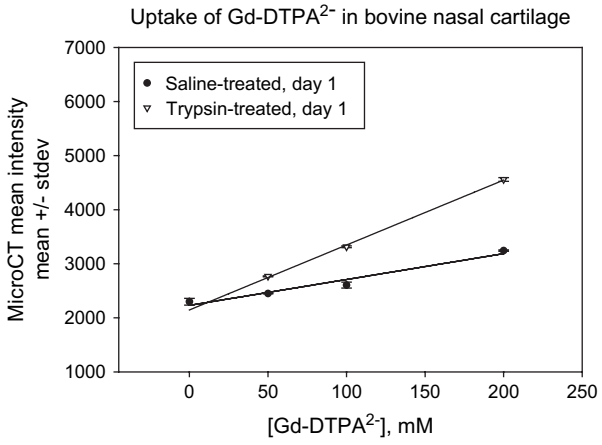

Fig. 3. Uptake of three concentrations of Gd−DTPA^{2-} in bovine nasal cartilage disks after pretreatment with saline or trypsin.

proteoglycans were depleted with trypsin pretreatment, increasing [Gd−DTPA^{2-}] also produced a linear response, but the slope was much greater (intensity = $2146 + 12.01 \times$ [Gd−DTPA^{2-}], $r^2 = 0.999$).

Figure 4 shows the dynamics of degradation of the cartilage disks with dilute trypsin as determined by the measurements of average intensity on microCT images. In those disks treated with Gd^{3+}, the intensity of the disks decreased with continued exposure to dilute trypsin, consistent with the hypothesis that the amount of Gd^{3+} present was proportional to the amount of negatively charged proteoglycans. In this study, the degradation appeared to be biphasic with a significant ~12% decline in intensity over the first 2 h of dilute trypsin exposure, followed by a 2-h plateau, and then by an additional significant ~4% loss of intensity over the final 2 h. Disks treated with the neutral agent, Gd−HP−DO3A, showed a slight, but significant ~4% increase in intensity over the first 2 h of exposure to dilute trypsin, possibly due to diffusion of the solution, rather than electrostatic attraction. Disks treated with Gd−DTPA^{2-} behaved oppositely from those treated with Gd^{3+}. There was a significant ~15% intensity increase that was maintained for the longer exposures to dilute trypsin. This fits the concept that more Gd−DTPA^{2-} penetrated a cartilage disk as negatively charged proteoglycans were removed by trypsin.

Proteoglycan was not detected in the disks treated with dilute trypsin and Gd−DTPA^{2-} or Gd−HP−DO3A. The reasons for this are not clear; however, it is possible that additional degradation due to residual trypsin could have occurred during the time prior to storage at −20°C. In contrast, proteoglycan was measurable in 10 of the 12 disks treated with saline or dilute trypsin and Gd^{3+}. (The two disks from which proteoglycan could not be detected were treated with dilute trypsin for 6 h.) Figure 5 shows the correlation between total proteoglycan content and total microCT intensity for 10 of the 12 samples exposed to Gd^{3+}. The relationship between the variables was very good ($r^2 = 0.81$). The large *y*-intercept was easily explained since Gd^{3+}-treated cartilage was still visible on microCT even after proteoglycan depletion.

Discussion

This work shows that gadolinium can be used to improve the appearance of cartilage in microCT images. This opens the possibility of measuring descriptors of cartilage morphology, such as thickness and volume, from microCT images. Furthermore, this work shows that there is a strong positive association between proteoglycan content and Gd^{3+} concentration in cartilage (Fig. 5). Since gadolinium attenuates X-rays, the intensity of cartilage treated with Gd^{3+} on microCT images reflects the proteoglycan content. Therefore, microCT studies of animal models of osteoarthritis, which heretofore have focused on bone[10], could be more comprehensive with the use of gadolinium treatment.

If Gd−DTPA^{2-} was completely repelled by negatively charged proteoglycans, a change in the microCT intensity of naïve disks should not have been observed with increasing [Gd−DTPA^{2-}]. However, Fig. 3 shows that Gd−DTPA^{2-} was taken up by naïve cartilage disks, albeit to a lesser extent than Gd^{3+}. The uptake of Gd−DTPA^{2-}, especially at the higher concentrations, indicated an incomplete

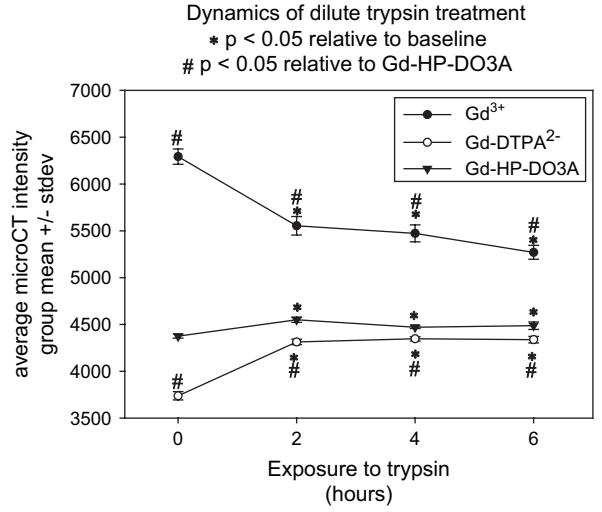

Fig. 4. The dynamic effects of trypsin exposure on uptake of 200 mM Gd^{3+}, 200 mM Gd−DTPA^{2-}, and 200 mM Gd−HP−DO3A as determined by microCT.

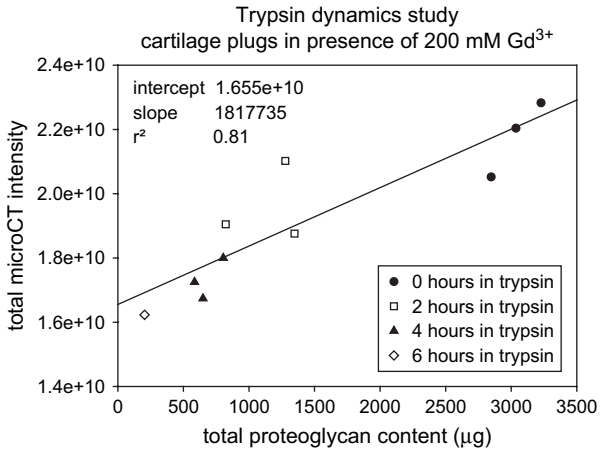

Fig. 5. Correlation between total intensity on microCT images and total proteoglycan content for cartilage disks exposed to 200 mM Gd^{3+}.

repulsion of the Gd–$DTPA^{2-}$ by the available proteoglycans; that is, there was an excess of Gd–$DTPA^{2-}$ that diffused into the cartilage. With proteoglycan depletion due to trypsin treatment, there was less repulsion and more Gd–$DTPA^{2-}$ could penetrate the cartilage, producing the greater slope seen in Fig. 3.

In the absence of trypsin pretreatment, there were fundamental differences in the image intensities of cartilage disks treated with Gd^{3+}, Gd–$DTPA^{2-}$, or Gd–HP–DO3A solution, as shown in Fig. 4. This cannot be explained by differences in gadolinium concentration, because these were identical for the solutions (200 mM). The duration of exposure seems an unlikely reason, as well, since the cartilage disks were soaked for 24 h with good mixing. The most likely explanation lies in the electrostatic charge and molecular structure of the three sources of gadolinium and their relative contributions. It was clear that the free gadolinium ion, Gd^{3+}, produced the greatest attenuation, most likely due to its charge and small size.

Of the disks pretreated with diluted trypsin, only those treated with Gd^{3+} had sufficient proteoglycan for measurement with the dimethylmethylene blue assay. The disks treated with diluted trypsin and Gd–HP–DO3A or Gd–$DTPA^{2-}$ behaved differently. Measurable amounts of proteoglycan were not found in any of these disks. This may indicate that Gd^{3+} quenches proteoglycan degradation by trypsin, whereas Gd–HP–DO3A and Gd–$DTPA^{2-}$ do not. Certainly the conditions for additional degradation were present after removal from the trypsin solution, since each disk was exposed to a gadolinium solution for 24 h at room temperature, followed by microCT scanning, which is known to warm the sample slightly during scanning.

The slope of the correlation between microCT intensity and proteoglycan content (Fig. 5) was probably a function of the concentration of the Gd^{3+} solution. A steeper slope could probably be obtained by using a greater concentration of Gd^{3+}. This, in turn, could increase the ability to distinguish between more subtle variations in proteoglycan content with microCT. However, the practicality of this would be limited by the solubility of $GdCl_3$.

The concentration of gadolinium needed for microCT studies of cartilage is open for debate and is governed by whether it is used *ex vivo* or *in vivo*. Certainly, as the concentration increases, the X-ray attenuation increases as well. This enables better visualization of cartilage and possibly improves the sensitivity to variations in proteoglycan content. Therefore, based on the work reported here, we have chosen a concentration of 200 mM Gd^{3+} for our ongoing studies of excised bones. Delivering such a concentration of Gd^{3+} to cartilage *in vivo* could be achieved by intra-articular or intravenous injection. The blood volume of rodents is about 60–70 ml/kg[11]. Therefore, to achieve a blood concentration of 200 mM Gd^{3+} requires approximately 12 mmol/kg. However, this would certainly be lethal, since the intravenous LD_{50} of $GdCl_3$ is approximately 0.5 mmol/kg in mice and rats[12,13]. By virtue of chelation, Gd–$DTPA^{2-}$ is much less toxic than Gd^{3+} with an intravenous LD_{50} of about 6 mmol/kg in mice and 8–10 mmol/kg in rats[13,14], but again a 200 mM blood concentration would not be acceptable. Therefore, microCT using a gadolinium probe will have its greatest applicability for terminal studies or studies of excised bones.

In summary, gadolinium treatment and microCT imaging are a powerful combination for studies of small animal bones, enabling the measurement of descriptors of bone and cartilage morphology, as well as proteoglycan distribution.

References

1. Ruegsegger P, Koller B, Muller R. A microtomographic system for the nondestructive evaluation of bone architecture. Calcif Tissue Int 1996;58:24–9.
2. Paulus MJ, Gleason SS, Easterly ME, Foltz CJ. A review of high-resolution X-ray computed tomography and other imaging modalities for small animal research. Lab Anim (NY) 2001;30:36–45.
3. Muller R, Koller B, Hildebrand T, Laib A, Gianolini S, Ruegsegger P. Resolution dependency of microstructural properties of cancellous bone based on three-dimensional mu-tomography. Technol Health Care 1996;4:113–9.
4. Kusaka Y, Grunder W, Rumpel H, Dannhauer KH, Gersonde K. MR microimaging of articular cartilage and contrast enhancement by manganese ions. Magn Reson Med 1992;24:137–48.
5. Bashir A, Gray ML, Burstein D. Gd–$DTPA^{2-}$ as a measure of cartilage degradation. Magn Reson Med 1996; 36:665–73.
6. Laurent D, Wasvary J, O'Byrne E, Rudin M. *In vivo* qualitative assessments of articular cartilage in the rabbit knee with high-resolution MRI at 3 T. Magn Reson Med 2003;50:541–9.
7. Zwicker C, Langer M, Urich V, Felix R. CT contrast administration of iodine, gadolinium and ytterbium. *In-vitro* studies and animal experiments. Rofo Fortschr Geb Rontgenstr Neuen Bildgeb Verfahr 1993;158: 255–259.
8. Schmitz SA, Wagner S, Schuhmann-Giampieri G, Wolf KJ. Evaluation of gadobutrol in a rabbit model as a new lanthanide contrast agent for computed tomography. Invest Radiol 1995;30:644–9.
9. Farndale RW, Buttle DJ, Barrett AJ. Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. Biochim Biophys Acta 1986;883:173–7.
10. Morenko BJ, Bove SE, Chen L, Guzman RE, Juneau P, Bocan TM, *et al*. *In vivo* micro computed tomography of subchondral bone in the rat after intra-articular administration of monosodium iodoacetate. Contemp Top Lab Anim Sci 2004;43:39–43.
11. Lee HB, Blaufox MD. Blood volume in the rat. J Nucl Med 1985;26:72–6.
12. Caille JM, Lemanceau B, Bonnemain B. Gadolinium as a contrast agent for NMR. AJNR Am J Neuroradiol 1983;4:1041–2.
13. Weinmann HJ, Press WR, Gries H. Tolerance of extracellular contrast agents for magnetic resonance imaging. Invest Radiol 1990;25(Suppl 1):S49–50.
14. Niendorf HP, Felix R, Laniado M, Schorner W, Claussen C, Weinmann HJ. Gadolinium–DTPA: a new contrast agent for magnetic resonance imaging. Radiat Med 1985;3:7–12.